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Enhanced Virus Replication in Mammalian Cells Exposed to Commercial Emulsifiers

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Mammalian cell cultures were used to show that a variety of commercial emulsifiers are capable of enhancing the sensitivity of these cells to infection with several viruses. Some emulsifiers were not active as enhancers, and those viruses that responded to the enhancing emulsifiers were single-stranded ribonucleic acid viruses. The double-stranded viruses that were tested were nonresponders.

Many popular insecticides are immiscible with water and require organic solvents and/or emulsifiers to aid in their dispersal as aqueous sprays. Earlier (2, 3) we showed the enhancing action of certain fenitrothion (FT) spray components on virus infections in mice. These reports noted that the increased incidence of mortality of FT spray-intoxicated mice (2) after infection with encephalomyocarditis (EMC) virus was probably due largely to emulsifiers. A number of these emulsifying chemicals have now been shown by us to enhance the sensitivity of a variety of cultured mammalian cells to several virus infections, but not to others.

MATERIALS AND METHODS

Cell cultures. The continuously cultured cell lines of African green monkey kidney cells (Vero) and mouse L-929 cells used in this study were originally obtained from K. MacCarthy, University of Liverpool, United Kingdom, and R. Stewart, Queen's University, Canada, respectively. HeLa cells were obtained from our own culture bank and originally from the American Type Culture Collection. They were passaged and stored in vials in liquid nitrogen. All cultures were grown in Eagle minimum essential medium (MEM) containing 10% fetal bovine serum (FBS; Flow Laboratories). The medium used to maintain mature monolayer cultures during treatment and afterwards was MEM containing 0.5% FBS. Dilutions of virus or emulsifiers were made in MEM alone.

Viruses. Stocks of vesicular stomatitis virus (VSV; Indiana strain), encephalomyocarditis (EMC) virus, vaccinia virus, *Herpesvirus hominis* type 1 (herpes-1), and reovirus type 2 (reo-2) were grown in L-929 cells, titered, and stored at −70°C in sealed vials. Poliovirus type 1 (polio-1) stocks were grown in Vero cells and similarly stored.

Insecticide and emulsifiers. FT (confirmed by us as >95% pure by gas chromatographic analysis), a solvent used for solubilization of FT (Aerotex 3470, Texaco of Canada), and two emulsifiers used widely in FT dispersal as an aerial spray (Toximul MP8, Charles Tennant & Co. Ltd.; Atlox 3409, Atlas Chemical Industry Ltd.) were obtained from commercial sources. Other emulsifiers were obtained either commercially or as gifts from the manufacturers, a list of which is given in Table 1.

Protocol for evaluating the effect of chemicals on the sensitivity of cell cultures to virus infection. Unless stated otherwise, cells were first grown to monolayer culture in 60-mm petri dishes (Falcon Plastics) as previously described (6, 7). They were then exposed in triplicate for 18 h at 37°C to various concentrations of components of commercial FT sprays, diluted in MEM, or to different emulsifiers. After this, their response to virus infection was evaluated and compared to that of the controls.

In the first series of experiments, three L-929 cell cultures were each exposed to concentrations of 0.1, 0.25, 1.0, 2.5, and 10.0 ppm of FT, Aerotex 3470, Toximul MP8, or Atlox 3409. Three additional cultures were kept in MEM alone for the required 18 h at 37°C. At this time, the treated and control groups of cultures were washed and inoculated with 0.3 ml of MEM containing an amount of VSV calculated to form a countable number of plaques in the control cultures. Adsorption was allowed to proceed, with occasional tilting to mix, for 90 min. At this time the culture was overlayed with 5 ml of 0.6% agarose in MEM containing 1% FBS and incubated for 2 days at 37°C. Virus plaques developing in the cultures were then identified, counted, and recorded (3, 4). The ratio of the average number of plaques in treated cultures compared to those found in control cultures was calculated and are listed in Table 2.

RESULTS

Effect of FT and FT spray components. The sensitivity of L-929 monolayer cultures to VSV infection after overnight exposure to various concentrations of FT and components of FT sprays was tested. The results (Table 2) show that FT does have a significant enhancing effect on the ability of cells to replicate virus and the solvent Aerotex 3470 has none. The major

TABLE 1. *Commercial name, manufacturer, and chemical nature of emulsifiers studied as enhancers of virus infection of mammalian cells*

Source	Emulsifier	Chemical nature
Charles Tennant & Co., Canada Ltd.	Toximul D Toximul R Toximul MP8 Toximul MP10	Anionic-nonionic blends of dodecylbenzenesulfonate/polyoxyethylene ethers (no information on proportions)
Wyandotte Chemical Corp.	Pluronic L31 Plurafac RA30 Pluronic L64	Nonionic polyalcoxyether polymer Polyoxyethylene alcohol Polyoxypropylene/polyoxyethylene copolymer
Imperial Chemical Industries	Brij 56 Atlox 3409	Polyoxyethylene cetyl ether Anionic-nonionic blend of dodecylbenzenesulfonate/polyoxyethylene ethers
Olin Corp.	Polytergent FL62	Polyalcoxyether, indeterminate composition
Richardson Co.	Richonate 408	Sodium dodecylbenzenesulfonate, anionic detergent
Ashland Chemical Co.	Varine 17	Surfactant (composition unknown)
Chemische Werke Hulf AG, West Germany	Marlophen 810	Surfactant (composition unknown)
Monsanto Chemical Co.	Sterox SL	Nonionic detergent $C_{14\text{-}15}O(CH_2CH_2O)_{12}H$
Shell Chemical	Nonidet P-40	Nonionic detergent $C_8H_{17}C_6H_4O(CH_2CH_2O)_9H$
Calbiochem	Triton X-100	Nonionic detergent $C_8H_{17}C_6H_4O(CH_2CH_2O)_{9\text{-}10}H$
Sigma Chemical Co.	Sodium dodecyl sulfate	Anionic detergent $C_{12}H_{25}NaO_4S$

TABLE 2. *The effect of 18 h of exposure to various concentrations of FT, the solvent Aerotex 3470, or the emulsifiers Toximul MP8 and Atlox 3409 on the susceptibility of L-929 cells to infection with VSV*

Compound	Ratio of plaques per treated culture/control culture[a] at concn (ppm):				
	0.10	0.25	1.0	2.5	10.0
FT	0.92	0.96	1.12	2.49	1.18
Aerotex 3470	0.90	0.94	0.76	0.96	0.74
Toximul MP8	0.78	0.88	1.15	1.88	4.19
Atlox 3409	0.82	0.88	1.05	2.92	3.60

[a] Average of three cultures per plaque count; average number of plaques in control cultures was 50.

enhancers were the emulsifiers Atlox 3409 and Toximul MP8.

Although these experiments were repeated several times using exposure times from 2 to 24 h, enhancement by the emulsifiers Toximul MP8 or Atlox 3409 could only be demonstrated with an exposure of at least 6 h. Exposures of 16 to 18 h at 37°C appeared to be optimum.

No enhancement could be demonstrated if exposure occurred at 4°C.

If virus and emulsifier were added simultaneously, no enhancement occurred and only the expected control number of plaques appeared in such treated cultures.

When cultures were treated with dilutions of a commercially mixed FT spray containing FT (7.5%), Aerotex 3470 (0.7%), and Toximul MP8 (0.7%), they responded with enhancement appropriate to the Toximul MP8 concentration and no synergistic effect was noted.

Response of different viruses to Toximul MP8. We next designed experiments to determine whether different viruses could participate in the enhancement phenomenon. Basically, the experiments were as described for VSV, with the exception that other virus inocula, containing a countable number of plaque-forming units, replaced VSV. L-929 cell monolayer cultures were exposed to 10 ppm of Toximul MP8 in MEM overnight for infection with VSV, EMC, vaccinia, herpes-1, and reo-2. Vero cells were similarly

exposed before infection with polio-1. After a wash in MEM, triplicate cultures were infected with a dose of appropriate virus, which was calculated to produce a countable number of plaques in control cultures. They were then incubated for the time required for plaques to develop: 2 days for VSV, polio-1, and EMC; 4 days for vaccinia and herpes-1; and 7 days for reo-2. Average plaque counts were recorded for both Toximul-treated and untreated control cultures, and the ratio of these plaque numbers was calculated as an index of enhancement. The data of one such experiment is given in Table 3, where it can be seen that the infectivity of single-stranded ribonucleic acid viruses (VSV, EMC, and polio-1) is enhanced, whereas the infectivity of double-stranded deoxyribonucleic acid viruses (vaccinia and herpes-1) and the double-stranded ribonucleic acid virus (reo-2) is not enhanced.

Effect of other emulsifiers. Our next series of experiments was designed to ascertain whether the enhancing activity was restricted to Toximul MP8 and Atlox 3409 or was shared by a wide variety of emulsifiers. An attempt was made to compare the chemical compositions of emulsifiers (Table 1) to see whether any similarities were present among good enhancers.

The experiments in which these emulsifiers were evaluated were performed in a similar manner to those in which the enhancing activity of Toximul MP8 and Atlox 3409 were observed. A change was made, however, in the type of cell culture. By comparison, we observed (8) that HeLa cells appeared more sensitive to the effects of Toximul MP8 than either Vero or L-929 cells. Consequently, HeLa cells were grown in monolayer culture and exposed to various dilutions of each emulsifier for 18 h at 37°C. They were then washed and infected with an amount of VSV calculated to produce a countable number of plaques. Average plaque numbers were calculated on the basis of three cultures per dilution. Untreated control cultures were exposed to diluent and similarly infected. An enhancement index was calculated for each emulsifier. This was simply the ratio of the average number of plaques found in emulsifier-treated cultures divided by the average number found in control, untreated HeLa cell cultures. Enhancement was shown to occur after exposure of cells to the Toximul series, Pluoronic L64, Brij 56, Atlox 3409, Polytergent FL62, Marlophen 810, and sodium dodecyl sulfate (Table 4). Eight other assorted emulsifiers did not cause enhancement.

DISCUSSION

There is little doubt that enhancement of virus infection by some emulsifiers is substantial. The induction of this effect requires that cultures be exposed for 4 to 6 h at 37°C before the cells respond. In addition, 4 to 6 h after the removal of the emulsifier, cells begin to return to the control state of lower permissiveness. We can assume, therefore, that certain metabolic events must occur in an exposed cell before enhancement can occur and that the effect is readily reversible.

From our small survey of many hundreds of available emulsifiers, it would appear that nonionic polymers of polyoxyethylene ethers are the most active. Mixtures of ionic surfactants and/or polyoxyethylene alcohols tend to be either inactive or of reduced activity.

The optimum concentrations for enhancement have been found to be just less than toxic. An increase in concentration above that found to be optimum for enhancement tends to be cytotoxic. This being so, one would expect that such doses of a cytotoxin would produce major and grossly detectable changes in cell morphology. However, as far as we could tell by light microscopy and thin-section electron microscopy, cells treated with an enhancing dose of emulsifier have no obvious structural abnormality.

Our experience with different continuous and primary cell types (8) has convinced us that emulsifier stimulation of host cell permissiveness

TABLE 3. *Effect of exposure of L-929 cells to 10 ppm of Toximul MP8 on the plaque efficiency of various viruses*

Virus	Plaque per culture[a]		Ratio
	Treated	Control	Enhancement index
VSV	218.7 ± 19.0	50.0 ± 1.0	218.7/50.0 = 4.37
EMC	36.3 ± 5.5	10.7 ± 1.2	36.3/10.7 = 3.39
Polio-1[b]	62.3 ± 1.5	36.3 ± 6.6	62.3/36.3 = 1.72
Herpes-1	42.3 ± 2.9	40.7 ± 4.5	42.3/40.7 = 1.04
Vaccinia	112.0 ± 16.0	106.7 ± 11.5	112.0/106.7 = 1.05
Reo-2	16.7 ± 3.8	16.0 ± 4.0	16.7/16.0 = 1.04

[a] Mean ± standard deviation.
[b] Experiment performed in Vero cells.

TABLE 4. *Plaquing efficiency of emulsifier-treated HeLa cells*

Emulsifier	Ratio of treated to control plaque no. at dilution (ppm) of:						Enhancer[a]
	100	50	10	5	1	0.1	
Toximul D	–	–	Toxic	8.0	1.8	1.0	+
Toximul R	–	–	Toxic	7.1	1.6	1.3	+
Toximul MP8	–	–	14.5	2.0	1.0	1.0	+
Toximul MP10	Toxic	Toxic	1.4	1.1	0.8	0.9	–
Pluronic L31	Toxic	1.1	0.8	0.8	0.7	0.7	–
Plurafac RA30	Toxic	1.2	1.6	0.8	1.0	0.8	–
Pluronic L64	–	–	Toxic	Toxic	11.0	0.5	+
Brij 56	–	–	Toxic	Toxic	9.9	1.6	+
Atlox 3409	–	–	3.6	1.9	1.1	0.8	+
Polytergent FL62	–	–	11.1	11.0	1.4	0.8	+
Richonate 40B	Toxic	1.1	1.4	1.4	0.7	1.1	–
Varine 17	Toxic	Toxic	1.7	1.3	1.4	0.7	–
Marlophen 810	–	–	Toxic	Toxic	12.7?	0.9	+
Sterox SL	Toxic	–	Toxic	–	0.8	1.0	–
Nonidet P-40	Toxic	–	1.5	–	1.3	1.1	–
Triton X-100	Toxic	–	1.2	–	1.1	1.0	–
Sodium dodecyl sulfate	Toxic	–	2.1	–	0.8	–	+

[a] We arbitrarily chose a doubling of sensitivity to indicate an enhancement.

to virus replication is a general phenomenon demonstrable in many mammalian cells. The same statement does not hold for the type of virus capable of participating in this phenomenon. Single-stranded ribonucleic acid viruses appear to be enhanced, whereas double-stranded ribonucleic acid and deoxyribonucleic acid viruses do not have their infectivity enhanced under our conditions. This specificity may have some interesting consequences if it can be demonstrated that it has significance in origination of diseases of multiple etiology.

Widespread exposure of humans to various chemicals and emulsifiers is a fact of modern life. The possible result of such exposure has been the subject of much speculation. Other investigators have noted the effect of certain chemicals on virus infectivity (1, 4, 5), and it is becoming increasingly suspect that in some diseases of multiple etiology an environmental factor apart from virus infection must play a part. In light of recent investigations, one group of chemicals in our environment that warrants further investigation should be the ubiquitous emulsifiers.

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